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Review

Tetrahydrobiopterin Deficiency: From Phenotype to Genotype*

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Summary

As a result of the selective screening worldwide during the last 18 years, approximately 250 patients with tetrahydrobiopterin deficiency were discovered. Most patients suffer from 6-pyruvoyl tetrahydropterin synthase deficiency (58%), followed by dihydropteridine reductase deficiency (35%), GTP cyclohydrolase I deficiency (3%), and "primapterinuria" (4%). The patients can be treated with neurotransmitter precursors, as well as with tetrahydrobiopterin. However, data on long term treatment are still scarce and it is therefore of great value to investigate all newborns with even mild hyperphenylalaninemia. Cloning of the enzymes involved in the biosynthesis and regeneration of tetrahydrobiopterin makes them to be easily accessible for biochemical and biological studies. So far, all proteins expressed heterologous are active in *E. coli*. Cloning of the wild type gene and mutant analysis of patients allow the rapid identification of the defective gene on the molecular level.

Key words: Tetrahydrobiopterin, Deficiency, Hyperphenylalaninemia, Gene cloning, DNA.

Introduction

Early detection of tetrahydrobiopterin (BH₄) deficiency became essential soon after it was recognized that a number of patients with hyperphenylalaninemia (HPA) show progressive neurological illness despite treatment with a low-phenylalanine diet (1, 2). The disease was initially named "malignant phenylketonuria (PKU)" because some of these patients died early. The first patients with BH₄ deficiency were described as "a genetic variant of PKU" (3) and were later characterized as suffering from a defect in the regeneration of BH₄. BH₄ is known to be the natural cofactor of three aromatic amino acid oxidase: phenylalanine-4-hydroxylase (PAH), tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase. PAH is responsible for the conversion of phenylala-

nine to tyrosine; tyrosine-3-hydroxylase and tryptophan-5-hydroxylase are the rate-limiting enzymes in the biosynthesis of catecholamines and serotonin, respectively (4). Thus, any defect in the biosynthesis or regeneration of BH₄ will result in a deficiency of biogenic amine neurotransmitters accompanied by HPA.

About 250 patients with BH₄ deficiency were discovered world-wide in the last 18 years (5, 6). Because of the different clinical and biochemical severity, this group of diseases was originally named "atypical PKU", a term which today is reserved for mild forms of PAH deficiency.

Although a wide range of metabolic defects may exist, the term "typical" or "atypical" form will be used according to the actual need to treat, or not to treat, the patients.

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*Dedicated to Professor Max Viscontini on the occasion of his 80th birthday.

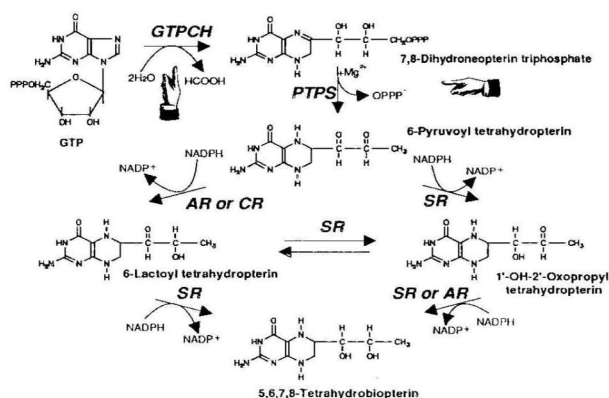


Figure 1. Biosynthesis of tetrahydrobiopterin including possible metabolic defects in hyperphenylalaninemia (13). GTPCH: GTP cyclohydrolase I; PTPS: 6-pyruvoyl tetrahydropterin synthase; SR: sepiapterin reductase; AR: aldose reductase; CR: carbonyl reductase.

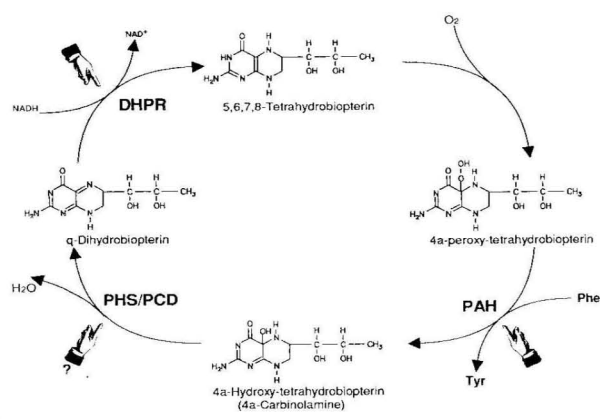


Figure 2. Phenylalanine hydroxylating system including possible metabolic defects in hyperphenylalaninemia (13). PAH: phenylalanine-4-hydroxylase; DHPR: dihydropteridine reductase; PHS/PCD: PAH stimulating protein/pterin-4a-carbinolamine dehydratase.

Biochemical observation

So far four molecular diseases have been known to cause HPA, including the classic form with a defect in the apoenzyme of PAH. Patients with the 3 variants suffer primarily from BH₄ deficiency, either by one of the two defects in the biosynthesis (Fig. 1) or one in regeneration (Fig. 2) of the cofactor (7). They are all inherited autosomal recessive.

In GTP cyclohydrolase I (GTPCH) deficiency (8), the enzyme catalyzing the first step in the biosynthesis of BH₄ is deficient and no pterins are synthesized. These patients are characterized by a neopterin and biopterin deficiency. Although it has been

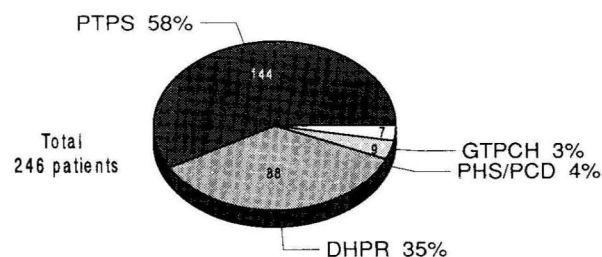


Figure 3. Results of the screening for tetrahydrobiopterin deficiency collected from the international register.

claimed that neopterin may play an active role in the cell-mediated immune response, cell proliferation, and differentiation, in one of these patients all humoral and cellular functions tested were found to be normal. In cerebrospinal fluid (CSF) neopterin, biopterin, isoxanthopterin, pterin, as well as the neurotransmitter metabolites, homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA), were generally low. This enzyme deficiency is rare, with 7 patients detected so far (Fig. 3).

6-Pyruvoyl-tetrahydropterin synthase (PTPS) deficiency (9) is the most common and heterogeneous variant with about 144 patients diagnosed all over the world, corresponding to about 58% of all patients with a BH₄ deficiency (Fig. 3). Patients with severe, partial or peripheral, and transient forms have been reported (10). 7,8-Dihydroneopterin triphosphate can not be converted to 6-pyruvoyl tetrahydropterin (Fig. 1), resulting in an accumulation of 7,8-dihydroneopterin triphosphate in tissue of these patients. This intermediate is readily dephosphorylated by pyrophosphatase and excreted as dihydroneopterin and its oxidation product, neopterin. High concentrations of neopterin, monapterin (isomer of neopterin), and 3'-hydroxysepiapterin, and only traces of biopterin are found in the urine of the patients with PTPS deficiency. In severe (typical) forms of PTPS deficiency the neurotransmitter status in the CSF is similar to that in patients with GTPCH deficiency. In the peripheral or partial (atypical) forms of PTPS deficiency, normal levels of neurotransmitter metabolites are measured. It is therefore of practical importance to find out whether or not the patient belongs to this type of PTPS deficiency and thus may need different treatment (see below). The transient form of PTPS deficiency may be due to a maturation delay of BH₄ biosynthesis.

In dihydropteridine reductase (DHPR) deficiency (11), the second most common form of BH₄ deficiency (88 patients detected; Fig. 3), quinonoid-dihydrobiopterin formed during the hydroxylation of phenylalanine to tyrosine accumulates, and the

amount of BH₄ synthesized on the *de novo* pathway is insufficient for the normal function of hepatic PAH (Fig. 2). Similarly, as in the case of PTPS and GTPCH deficiencies, these patients are mostly characterized by a depletion of catecholamines and serotonin (12). Since quinonoid-dihydrobiopterin tautomerized readily to 7,8-dihydrobiopterin, patients with DHPR deficiency excrete high amounts of total biopterin (7,8-dihydrobiopterin and biopterin). In addition, owing to the lack of BH₄ in these patients, there is no feedback inhibition of GTPCH and thus neopterin biosynthesis is generally activated.

Primapterinuria is the recently discovered variant of HPA characterized by the excretion of the 7-substituted pterins, primapterin (7-biopterin) and anapterin (7-neopterin) in the patient's urine (13, 14). Primapterinuria differs from classic PKU and other variants of BH₄ deficiency because obviously these patients do not need any treatment. Nine patients have been detected so far (Fig. 3). The first patients were described by Dhondt *et al.* (15) and Blaskovics and Giudici (16). A loading test with BH₄ was the first indication that primapterin may derive from biopterin (17). Furthermore, 7,8-dihydrobiopterin and sepiapterin given orally resulted in a significant increase in biopterin and primapterin, whereas the ratio remained between 1.0 and 1.5 after loading (18). From *in vitro* experiments it is known that primapterin can be formed during the PAH reaction in a phenylalanine-4-hydroxylase-stimulating protein (PHS)/pterin-4a-carbinolamine dehydratase (PCD) free system (Fig. 2) (19, 20). So far, all known enzymes involved in the biosynthesis or regeneration of BH₄ in these patients have been found to be normal and a deficiency of PHS/PCD has been proposed. However, PAH and PHS/PCD, per se, have not been measured.

Clinical observation

Typical forms

The clinical course of the illness in untreated patients is similar in typical (severe) PTPS, DHPR and GTPCH deficiencies (10). The median age at which clinical signs become evident is 4-5 months, but symptoms do not necessarily correlate with age of diagnosis, even in the same family. However, when information on the neonatal period is available, abnormal signs (poor sucking, decreased spontaneous movements, floppy baby) can be noticed during the neonatal period (6).

The common symptoms are mental retardation,

convulsions (grand mal or myoclonic attacks), disturbances of tone and posture, drowsiness, irritability, abnormal movements, recurrent hyperthermia without infection, hypersalivation, swallowing difficulties. Diurnal fluctuation of alertness and neurologic symptoms are also reported. Microcephaly is observed in both diseases but with a higher incidence in PTPS deficiency (52%) than in DHPR deficiency (33%). However, in 13 out of 25 DHPR deficient patients for whom repeated measurements with increasing age were available, evolution of head circumference to progressive microcephaly was observed, whether patients were treated or not.

Atypical forms

Atypical PTPS deficiency: The absence of clinical signs theoretically defines atypical forms. However, in 2 cases neonatal hypotonia was noticed, 2 were mentally retarded, 1 had focal signs on the EEG, and 1 presented with acute but transient symptoms (behaviour problems, neurovegetative signs, sleeping difficulties).

Atypical DHPR deficiency: Some authors have suggested that partial deficiency of DHPR activity may also exist. That may explain a few reports on patients who were clinically unusual in some aspects (21). *Primapterinuria:* Although this form seems to have no serious consequences, minor abnormalities can be observed. In the first case (15), slight tremors of upper limbs after stimulations and a moderate tendency to hypertonia were noticed during the neonatal period. An EEG at 3 weeks of age revealed a few spikes and sharp waves in the frontal area. With dietary control of blood phenylalanine, neurological development normalized. In another case reported by Blaskovics and Giudici (16), transient hypotonia and motor delay were also noticed.

Treatment

The goals of the treatment are to control HPA by dietary restriction of phenylalanine or BH₄ administration, and to restore neurotransmitter homeostasis by oral administration of amine precursors.

Neurotransmitter replacement

L-Dopa and 5-hydroxytryptophan (5-HT) administration represents a common therapeutic approach to BH₄ deficiencies. Carbidopa, an inhibitor of peripheral aromatic amino acid decarboxylase, reduces the therapeutic dose of L-Dopa.

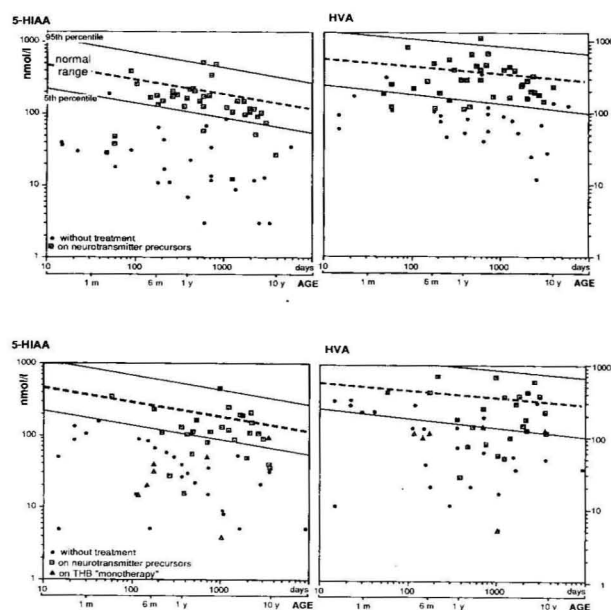


Figure 4. CSF levels of 5HIAA and HVA of patients with DHPR deficiency (upper) and PTPS deficiency (lower). ● without treatment; □ on neurotransmitter precursors; △ on BH₄ "monotherapy"; shaded area represents normal range (percentile 5-95) established from the distribution of 292 CSF determinations in normal controls.

The monitoring of treatment represents the crucial point. Unfortunately, there are no biochemical parameters measurable in the periphery which would allow the control of treatment. The analysis of neurotransmitter metabolites in CSF represents the only way to follow up the efficiency of treatment (Fig. 4). However, such an investigation cannot be easily repeated. Two cases (Fig. 5) illustrate the need of a balanced administration of Dopa and 5-HT.

In practice, the optimal dosage can only be found on a clinical basis and should be adjusted to the requirement of each patient with monitoring for adverse effects (22, 23) and disappearance of neurological symptoms. Diurnal fluctuations are often observed and may require changes in the schedule of drug administration. Difficulties with parents for compliance with treatment are frequently reported because of the constant need of treatment adjustments, which requires excellent collaboration with the family.

Control of blood phenylalanine levels

Although BH₄ deficient persons exhibit a higher dietary phenylalanine tolerance than classical PKU patients, a limiting factor in the response to neurotransmitter precursor therapy might be the plasma

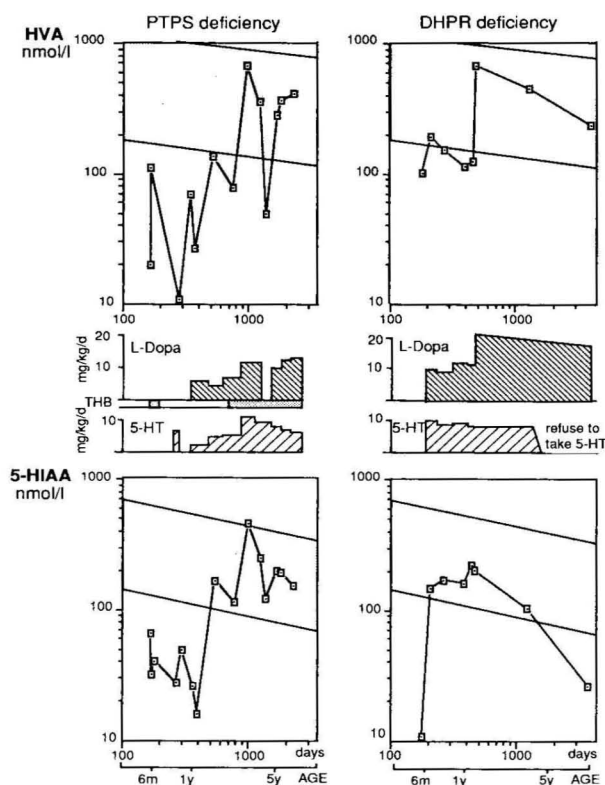


Figure 5. CSF monitoring of the treatment with neurotransmitter precursors.

phenylalanine fluctuations, which could alter the dose-effect relationships of these substances by interfering with their membrane transport or by competitive inhibition of tyrosine and tryptophan-hydroxylase (12). The control of blood phenylalanine concentrations has to be stricter than in other HPAs. Some patients on neurotransmitter treatment have had neurological problems with phenylalanine concentrations as low as 360 μ mol/l.

Tetrahydrobiopterin administration

In PTPS deficiency, evidence of a bloc in BH₄ synthesis invited attempts to treat these patients with BH₄ replacement alone (so-called BH₄ monotherapy). The main advantage was that BH₄ replacement would not bypass any regulatory mechanisms that normally control neurotransmitter synthesis and secretion. However, in most cases BH₄ monotherapy failed to restore a normal level of cerebral neurotransmitter synthesis as confirmed by low concentrations of 5HIAA and HVA in the CSF (Fig. 4). On the other hand, the use of BH₄ to control blood phenylalanine levels appears to be efficient and is recommended. Relatively low doses of BH₄ normal-

ize blood phenylalanine levels and offer an interesting alternative to a phenylalanine-restricted diet.

In DHPR deficiency, BH₄ cannot be given in sufficient amounts to maintain an adequate pool of BH₄ in the absence of the endogenous reducing system sustained by DHPR.

Folinic acid supplementation

The hypothesis of a gradual CNS folate deficiency in DHPR deficient patients is supported by low CSF folate levels, and also by the frequent occurrence of calcifications in the basal ganglia similar to those described in congenital folate malabsorption or in methotrexate toxicity. A direct benefit of folinic acid supplementation has been clinically demonstrated (24).

The clinical outcome

The replacement of neurotransmitters has been proven to be beneficial, though incomplete. Because in many cases the treatment was administered late, resolution of some of the major neurological signs gave the impression of a "remarkable" improvement, as initially published. Even when patients reached milestones for age, speech difficulties and sleeping problems were reported. Two PTPS and 6 DHPR deficient patients died although the treatment had been started within the first month of life.

Although results of a larger group of patients treated early might temper these pessimistic conclusions, four reasons can explain the limited success of treatment:

1. The possibility of fetal brain damage is likely to occur, especially in PTPS deficiency, since low birth weight, clinical signs, and microcephaly at birth are often reported.
2. Possible iatrogenic damage of neurotransmitter precursors cannot be excluded.
3. It can also be speculated that the quality of the response to therapy depends on the severity of the metabolic defect, as illustrated by the better response of patients with near normal CSF HVA concentrations at diagnosis (25). In DHPR deficiency, it has also been suggested that CRM⁺ mutants may have a better prognosis (26).

Do atypical forms have to be treated?

These forms theoretically do not require treatment. Besides this affirmation, the decision to treat or not

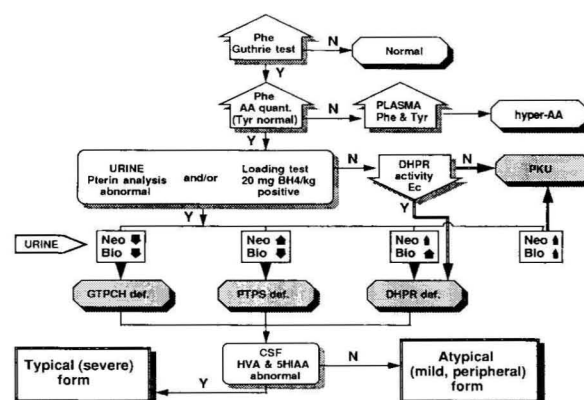


Figure 6. Screening policy and the diagnostic flow chart in the differentiation of hyperphenylalaninemia variants. ↑ represents increased value; ↓ represents diminished value. Phe: phenylalanine; Tyr: tyrosine; AA: amino acids; Neo: neopterin; Bio: biopterin; Y: yes; N: no.

is not clear-cut. With regard to the obvious heterogeneity and unclear prognosis it seems adequate to treat newborns with the so called "atypical" or "peripheral" form, at least with BH₄, and to monitor their development carefully.

Screening for BH₄ deficiency and differential diagnosis

Screening for BH₄ deficiency should be done in all newborns with even slight hyperphenylalaninemia (plasma phenylalanine < 400 μmol/l) as well as in older children with neurological symptoms (27, 28). The following protocol should be followed:

1. Analysis of pterins in urine
2. Measurement of DHPR activity in blood from Guthrie cards
3. Analysis of phenylalanine in plasma before and after the BH₄ loading test

The first two tests are essential and allow to differentiate between all variants of BH₄ deficiency. With some limitations, the BH₄ loading test is an additional useful diagnostic tool for the rapid discrimination between the classic PKU and biopterin variants. The diagnostic flow-chart is shown in Fig. 6.

Analysis of pterins

HPLC is the method which is used in most laboratories to analyze pterins in urine (29, 30). The analysis must be performed at elevated plasma phenylalanine levels and not under low-phenylalanine diet. Either a native urine or urine dried on filter

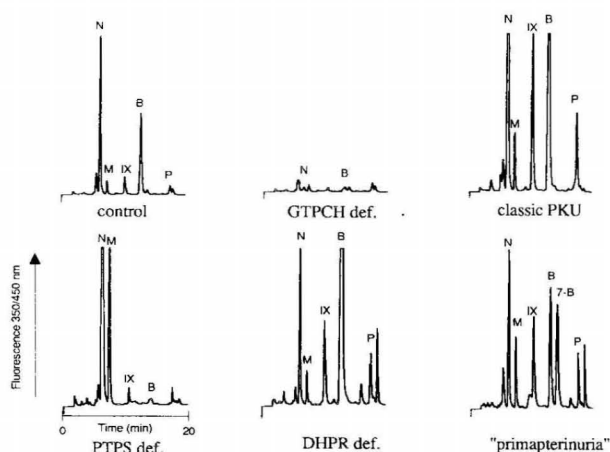


Figure 7. HPLC of urinary pterins (oxidized) from a healthy control and patients with different variants of hyperphenylalaninemia. N: neopterin; M: monapterin; IX: isoxanthopterin; B: biopterin; 7-B: primaapterin; P: pterin.

paper (31) may be used for the selective screening. The concentrations of pterins are based on creatinine levels, which makes a urine collection unnecessary. A characteristic urinary pterin pattern makes it possible to identify all four variants of BH_4 deficiency (Fig. 7).

In GTPCH deficiency, neopterin and biopterin excretion is extremely low (below 10% of normal) while the percentage of biopterin (of the sum of neopterin plus biopterin) is approximately 50% (neopterin/biopterin ≈ 1). Patients with PTPS deficiency excrete trace amounts of biopterin and high amounts of neopterin (biopterin < 5%; neopterin/biopterin = 40–300). Excretion of pterins is not always informative for DHPR deficiency. High biopterin concentrations (biopterin > 80%) and normal or slightly increased neopterin concentrations are rather characteristic for older children suffering from DHPR deficiency and particularly in very young patients may be almost normal or close to values found in patients with classic PKU. In patients with primaapterinuria the pterin pattern is similar to that in patients with PTPS deficiency (elevated neopterin and low biopterin; neopterin/biopterin = 18–36) in the very early neonatal period. With increasing age neopterin normalizes while the excretion of primaapterin persists and the ratio of biopterin to primaapterin remains close to 1.

Loading test with BH_4

The loading test with BH_4 allows the detection of all HPA variants with a defect of the BH_4 biosynthesis, even if the defect is only partial. A rapid

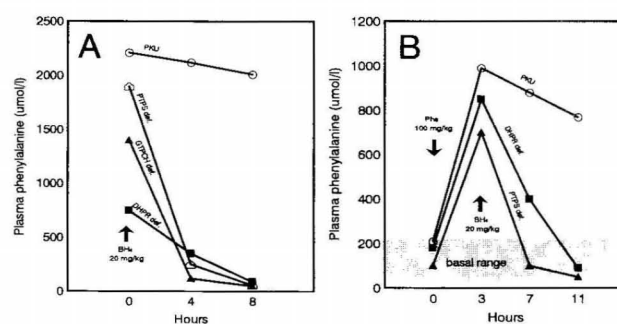


Figure 8. Typical results of a simple (A) and combined (B) tetrahydrobiopterin loading tests in patients with hyperphenylalaninemia.

normalization of the elevated plasma phenylalanine level occurs within 4 to 8 hours after administration of BH_4 (Fig. 8A). The test was originally introduced by Niederwieser *et al.* (32) using 7.5 mg of BH_4 /kg bw. However, it was soon noted that this procedure may fail in some patients with DHPR deficiency (26), namely those belonging to the CRM^+ (cross reactive material positive) type having a mutant enzyme. This limitation can be overcome by increasing the loading dose up to 20 mg BH_4 /kg bw. However one patient, described as a nonresponder even at high dose of BH_4 , died despite early diagnosis and treatment and may represent a "severe" form of DHPR deficiency (33).

An alternative procedure for the screening of hyperphenylalaninemia is the combined phenylalanine (100 mg/kg bw) and BH_4 (20 mg/kg bw) loading test (34). This test takes advantage of being performed while patients are on a phenylalanine-restricted diet, particularly when pterin analysis is not available. As for the conventional loading test, this also has to be combined with the measurement of DHPR activity in blood. The decrease of serum phenylalanine 4 and 8 hours after BH_4 administration (7 and 11 hours after phenylalanine administration) allows differentiation between PTPS and DHPR deficiencies and classic PKU (Fig. 8B).

Molecular biology of BH_4 biosynthetic and regenerating enzymes: gene cloning and expression

Cloning of the genes or their corresponding cDNAs encoding the enzymes for BH_4 metabolism will have many beneficial consequences such as the possibility to overproduce the proteins and purify them in large quantities for enzymatic and structural studies, and antibody production. In addition, for the defects described for the four enzymes involved

in BH₄ metabolism (see Figs. 1 and 2), identification of mutations on the DNA, responsible for aberrant expression or activity in patients, is the basis for future understanding of the deficiency on a molecular level.

GTPCH, the first biosynthetic enzyme for BH₄, has been cloned and DNA sequence information is available from two different sources: rat liver (35) and *Escherichia coli* (36, 37). The isolated rat liver cDNA codes for a putative protein of 241 amino acids, whereas the mature protein has the first 11 amino acid residues deleted. Thus, posttranslational modification produces a protein of 230 amino acid residues with a molecular mass of 25,784 Da. Expression of the rat GTPCH cDNA in *E. coli* was reported to result in an active enzyme, indifferent whether it was expressed as the premature form (complete cDNA) or as the mature form (38). The mature *E. coli* GTPCH contains 222 amino acid residues with a molecular mass of 24,742 Da. For this enzyme, only the starting methionine was found to be removed. An amino acid alignment of the rat and *E. coli* sequences revealed homology extending over the entire length of the proteins. Overproduction and purification of recombinant GTPCH from *E. coli* allowed the crystallization and a preliminary characterization of the X-ray structure (39). Although a fragment coding for the human GTPCH was reported to be cloned (39), isolating of the complete coding region is eagerly awaited in order to study defects in patients with reduced GTPCH activity.

The PTPS is the most frequent enzyme activity that is reduced or missing in patients exhibiting a BH₄ deficiency. The rat and human liver cDNAs have been cloned and sequenced (40-42). The rat cDNA codes for a protein of 144 amino acids. The first four amino acids are not present in the purified enzyme, resulting in a mature rat protein with a molecular mass of 15,855 Da. The human liver cDNA codes for a protein of 145 residues with an estimated molecular weight of 16,387. It exhibits a 82% identity to the rat amino acid sequence. Since the amino terminus of the human PTPS was not accessible by Edman degradation due to end blocking, the N-terminus of the mature protein is not known. Both, the rat and human cDNA can be expressed as an active enzyme in *E. coli* that makes the PTPS easily accessible for further analysis. This powerful system made it possible to identify the first mutations in the coding region for PTPS, responsible for a lowered enzymatic activity in patients with BH₄ deficiency (41, Thöny *et al.*, unpublished).

A cDNA for SR, the enzyme catalyzing the final step in the BH₄ biosynthesis, was isolated from rat

and human liver (43, 44). The rat liver cDNA encodes a polypeptide of 262 amino acid residues with a molecular mass of 28,169 Da, including the N-terminally acetylated methionine (45). A corresponding cDNA isolated from human liver codes for a protein of 261 amino acid residues with a molecular mass of 28,047 Da. A sequence comparison revealed a 74% identity between these two enzymes. The rat liver enzyme was expressed in *E. coli* as a translational fusion to the N-terminal fragment of β -galactosidase. Since this resulted in an active SR, it is expected that also the human enzyme, expressed as a non-fusion protein in *E. coli*, will yield SR activity.

Alternative reductases to SR, such as AR and CR, were found to accomplish at least *in vitro* the two step reduction of the carbonyl groups (compare with Fig. 1; (46)). These alternative pathways might account *in vivo* for the fact that so far no SR deficiency was observed in man. Human cDNAs for AR and CR have been cloned from several tissues but will not be discussed in this article (47-50).

Following the oxidation of BH₄ by the PAH, the pterin cofactor is proposed to be efficiently recycled in the presence of two proteins, the PHS/PCD and DHPR (Fig. 2). Based on the elucidation of the complete amino acid sequence of the human and rat PHS/PCD (51), the corresponding cDNA was cloned from rat and human liver (Thöny *et al.*, unpublished). The native protein was found to be identical in rat and human, and consists of 103 amino acids with a molecular mass of 11,909 Da. The N-terminally encoded methionine is cleaved off, and the second amino acid, the alanine residue, was found to be acetylated in the mature protein. Heterologous expression of the cDNA in *E. coli* yielded the activity in a crude extract, thus allowing the purification of the protein in large amounts and making it accessible to refined biochemical studies. Unexpectedly, the PHS/PCD was found to be identical to the hepatic nuclear dimerization factor for the transcription factor HNF-1 α (52, Thöny *et al.*, unpublished). To clarify the meaning of this finding, it will be helpful to have recombinant protein available, free from any contamination, to perform *in vitro* studies with PAH. In addition, the newly described form of hyperphenylalaninemia characterized by 7-pterin excretion was proposed to be due to a defect in PHS/PCD activity (13-17). The isolation of the DNA encoding this protein will allow to further investigate the enzymatic defects in such patients on the molecular level.

The enzyme responsible for the completion of the reductive recycling of BH₄, DHPR, is thus far the protein best studied biochemically and genetically.

The DNA sequence encoding the DHPR is available from rat and human liver (53-55). The rat liver cDNA codes for 241 amino acids. However, the mature protein appears to have the initiating methionine cleaved off and an acetylated alanine residue on its N-terminus. The molecular weight of 25,420 (240 amino acids) is very similar to that of the human liver enzyme and a sequence alignment shows that the two proteins differ only in 10 amino acid residues. The human DHPR protein has a length of 244 amino acids and a molecular mass of 25,760 Da (serine at position 51, (ref. 54)) or 25,774 Da (threonine at position 51, (ref. 55)). The human enzyme was also reported to be N-terminally blocked due to acetylation, however, the mature end of the protein is not known. Heterologous expression of the entire cDNA encoding the human DHPR has been reported and yielded the active enzyme in *E. coli* (56). Moreover, the rat liver DHPR was crystallized and its 3-dimensional structure was elucidated (57). Interestingly, it is structurally and mechanistically different from the dihydrofolate reductase. Using PCR technology, mutations on the DNA level were detected that caused a change in the polypeptide chain (21, 58). Also, RFLP was shown to be a useful tool for prenatal diagnosis of DHPR deficiency (59).

In summary, it is remarkable that probably all the human metabolic enzymes for BH₄ biosynthesis and recycling appear to be active when expressed in *E. coli*. Mutations in GTPCH, PTPS, PHS/PCD or DHPR can now be easily detected for their effects on enzyme activity by expressing them in *E. coli*. At the same time, the purification of native and also mutant enzymes in large amounts will be facilitated. Cells from patients or patients cultured fibroblasts contain insufficient material to isolate enzymes for such studies. Using PCR-based technology, prenatal diagnosis for BH₄ deficiency might be introduced as a noninvasive method without any risk for the offspring. In the future, one might even consider a completely new area of treatment by using gene therapy, i.e. transferring the wild-type gene into patients with a BH₄-deficiency in order to restore the defective gene. Such an approach was shown to be feasible *in vitro* for cells derived from patients with a defect in the DHPR gene (60).

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